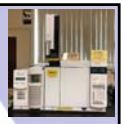
SAS4305 Chemical Technology for Chemical Industry



Chapter 9
Liquid Chromatographic Techniques (液相色譜技術)

References:

- Skoog, D.A., Crouch, S.R., Holler, F.J., West, D.M. (2014). Fundamentals of Analytical Chemistry, 9th edition, Brooks/Cole, Chapter 33.
- Skoog, D.A., Holler, F.J., Crouch, S.R. (2018). Principles of Instrumental Analysis, 7th edition, Thomson, Chapters 28.

Table of Content

2

Intended Learning Outcomes

Upon completion of Chapter 9, you are able to .....

- Explain the working principles of high performance liquid chromatography (HPLC);
- Explain the instrumentation of HPLC;
- Select and justify correct choice of stationary, mobile phases and detectors;
- Realize qualitative and quantitative analysis of HPLC.

3

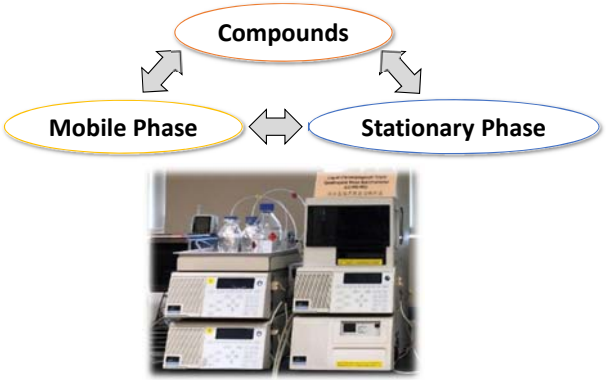
9.1 Working Principles of HPLC

- A technique for the separation of:
  - ***thermally unstable*** 熱力不穩定 and
  - ***non-volatile***非揮發性***samples*** (***usually polar compounds***).
- ***Liquid samples*** are injected into the liquid chromatography, the samples are then pass through the column 柱子 for separation 分離 ***under high pressure*** 高壓力
- Mobile phase (*organic / inorganic solvent*) **carries liquid samples** (*analyte + matrix*) **passes through column**.
- **Retention** 保留 **of analyte** (or matrix) by column **stationary phase**.
- **Elution** of analyte / matrix followed by **signal detection**.

4

9.1 Working Principles of HPLC

- Solute **distributes** (*interacts*) between mobile phase and stationary phase **based on** different **polarities** 極性 and relative **solubilities** 溶解度.

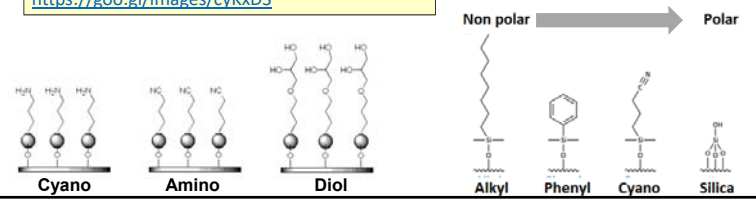


5

9.1.1 Normal Phase HPLC

- Using **polar stationary phase** (*e.g. silica*) & **non-polar mobile phase** (*e.g. hexane*)
- **NOT** generally chosen ***for polar analytes*** due to their high affinity for stationary phase, resulting in very **long retention times**.
- Then, the **NON-polar compound comes out first**.

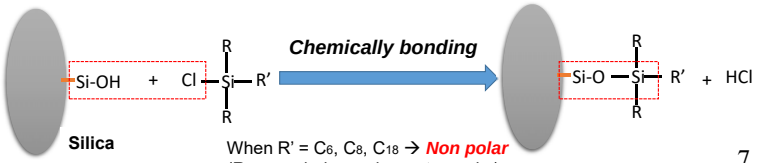
Mobile phase and column in Normal Phase HPLC
https://goo.gl/images/cyKxDS



9.1.2 Reserve Phase HPLC

- Using **non-polar stationary phase** (*e.g. C18*) & **polar mobile phase** (*e.g. methanol, ACN, water*).
- Applied to separation of **polar / semi-polar analytes**.
- A **hydrophobic molecule** (疏水分子) (*alkane C18*) is **chemically bonded** on to the silica surface.
- Then, the **MOST polar compound comes out first**.

Mobile phase and column in Reserve Phase HPLC
https://goo.gl/images/sF7TM8



When R' = C_6, C_8, C_{18} → ***Non polar***
(Reversed-phase chromatography)

7

Normal Phase and Reserve Phase HPLC

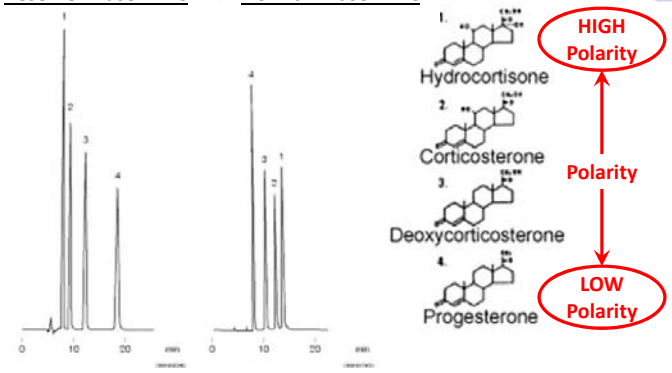
★ Classwork

Compare the Normal phase and Reserve phase HPLC.

	Normal phase HPLC	Reserve phase HPLC
Stationary phase		
Example:		
Mobile phase		
Example:		
Elution order		
Analytes		

Normal Phase and Reserve Phase HPLC

Reserve Phase HPLC **Normal Phase HPLC**



http://quimica.udea.edu.co/~carlopez/cromatohplc/fareversa-io-80.jpg

9

Normal Phase and Reserve Phase HPLC

Reserve Phase HPLC

high polarity → low polarity

Elute first

Shorter retention time

Normal Phase HPLC

low polarity → high polarity

Elute first

Shorter retention time

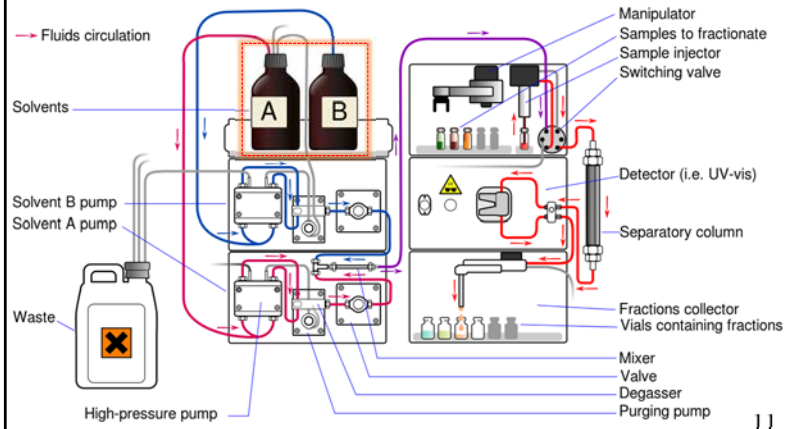
Elution order / retention time of different polarity compounds

http://quimica.udea.edu.co/~carlopez/cromatohplc/fareversa-io-80.jpg

10

9.2 Instrumentation of HPLC

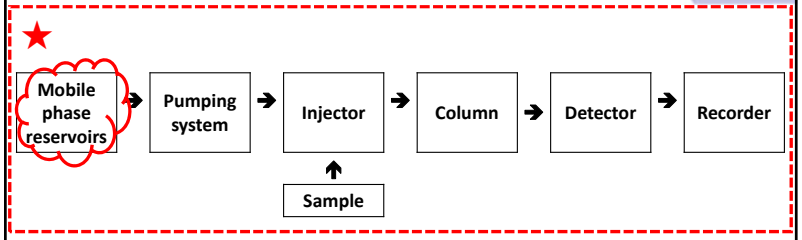
Components of HPLC https://goo.gl/images/CaAPtm



11

9.2 Instrumentation of HPLC

Major components (instrumentation) of a HPLC



12

9.2.1 Mobile phase reservoirs

- **Dissolved gases / dust** must be **removed** from mobile phase:

TWO methods to move dissolved gases / dust:
1. De-gassing (*by ultrasonic* 超聲波 *bath*);
2. Filtering (*by filter*).


Solvent reservoir


Ultrasonic bath


Filtration setup

13

9.2.1 Mobile phase reservoirs

Isocratic elution (平等沖提):

- Using **single** 單一 **solvent / solvent mixture**,
- keeping **composition** 成分 **of mobile phase constant** 不變

- **Polarity**極性of solvent does **NOT change** during separation.

https://goo.gl/images/TgVpAQ

14

9.2.1 Mobile phase reservoirs

Gradient elution (坡度沖提):

- Using **solvent mixture**,
- **composition**成分**of mobile phase varies** 改變 during elution.
- **Polarity** 極性 of solvent **changes** during separation.
- Gradient elution can **improve separation efficiency**.

https://goo.gl/images/j6sb92

15

9.2.1 Mobile phase reservoirs

Mobile phase (isocratic and gradient)

Isocratic mobile phase 平等沖提	**Gradient mobile phase 坡度沖提**
• "iso" - mean same • the mobile phase stays same for the entire run	• the mobile phase composition **changes throughout the run**
For example: • running time is 10 minutes, 80% methanol & 20% water for the run • running time is 20 minutes, 100% acetonitrile for the run	For example: Time (Min) / Mobile phase A (Methanol) / Mobile phase B (Water) 0: 80% / 20% 5: 60% / 40% 10: 40% / 60% 13: 20% / 80% 15: 80% / 20% 20: 80% / 20%

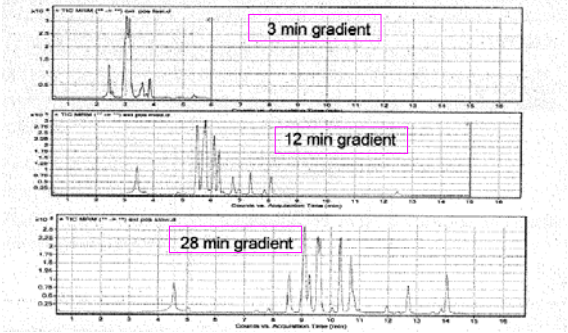
16

9.2.1 Mobile phase reservoirs

Reference ONLY!!

Mobile phase (isocratic and gradient)

LC/MS/MS Analysis of mycotoxins extracted from spiked corn using 3 different gradient elution times



17

9.2.1 Mobile phase reservoirs

Reference ONLY!!

Mobile phase (isocratic and gradient)

Recovery for 31 Mycotoxins from spiked corn using 3 different LC/MS/MS gradient analysis conditions

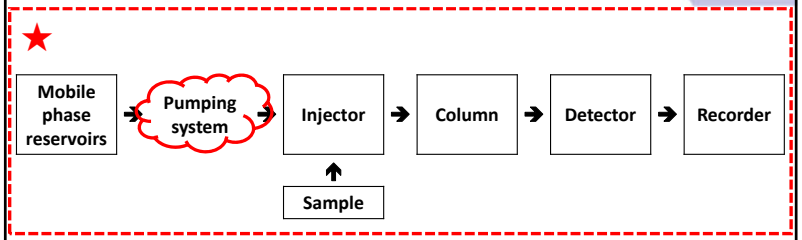
mycotoxins	3 min gradient	12 min gradient	28 min gradient	mycotoxins	3 min gradient	12 min gradient	28 min gradient
Aflatoxin B1	94.4	93.3	97.1	nivalenol (NIV) (ESI)	93.9	99.1	97.3
Aflatoxin B2	43.8	85.1	85.5	neosolaniol (NEO)	37.0	74.3	94.3
Aflatoxin G1	41.9	79.6	89.3	T-2 triol	19.2	74.4	71.4
Aflatoxin G2	48.8	77.6	106.8	T-2 tetraol (ESI)	77.4	94.7	94.6
Aflatoxin M1	49.1	77.0	00.0	deepoxy-deoxynival	6.8	72.6	76.8
zearelenone	112.2	94.7	99.4	fumonisin B1	140.0	110.7	91.9
patulin	2.0	98.0	95.3	fumonisin B2	82.3	86.6	80.9
citrinin	46.2	80.6	89.3	sterigmatocystin	79.4	84.1	100.0
fusarenon-x (Fus-x) (ESI)	66.1	85.3	96.8	ergocornine	75.4	87.2	98.8
deoxynivalenol (DON) (ESI)	56.0	84.0	106.7	ergocristine	90.4	102.0	93.3
3-acetyl DON	30.0	86.7	89.3	ergocryptine	76.9	84.6	94.1
15-acetyl DON	48.3	77.0	86.0	ergometrine	66.6	86.7	92.9
HT-2 toxin (HT-2)	53.3	68.5	88.2	ergosine	73.1	96.0	91.9
diacetoxyscirpenol (DAS)	88.6	81.5	74.3	ergotamine	70.8	82.9	92.7
T-2 toxin (T-2)	54.8	96.7	93.6	ochratoxin-A	192.3	88.9	97.7
				ochratoxin-B	96.2	96.0	92.0
Average % recovery 31 toxin	66.1	86.0	91.8				
% std dev	37.4	10.6	8.6				

Conditions: 1 g corn spiked with 20-160 ng/g of each mycotoxins, extracted with 2 ml of 80% ACN in water with 0.025% TFA for 10 min, centrifuged and filtered (30K MWCO filter), then diluted with 1.5 ml of water and 10 ul injected onto the LC/MS/MS. Separation used poroshell 120 2.1x50 mm 2.7 um particles gradient 5-95% ACN in 3, 12 and 28 min, 0.3 ml/min, ESI pos ion detection used 0.025% TFA and ESI neg ion detection used 20 mM AM Act.

18

9.2 Instrumentation of HPLC

Major components (instrumentation) of a HPLC

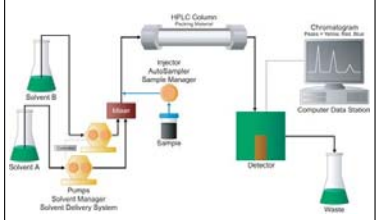
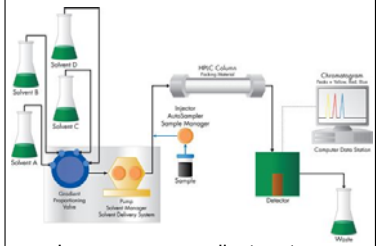


19

9.2.2 Pumping System

Reference ONLY!!

Pumping System

High-pressure gradient system	Low-pressure gradient system
• Consists of more than one pump	• Consists of one pump, the solvents are mixed first
	
High-pressure gradient system	Low-pressure gradient system
https://goo.gl/images/6ch81q	https://goo.gl/images/pMyDdq

20

9.2 Instrumentation of HPLC

Major components (instrumentation) of a HPLC

★

Mobile phase reservoirs → Pumping system → Injector → Column → Detector → Recorder

Sample

1. Manual injection - Sample loop

2. Auto-injector

21

9.2.3 Injection System

Reference ONLY!!

Injection System

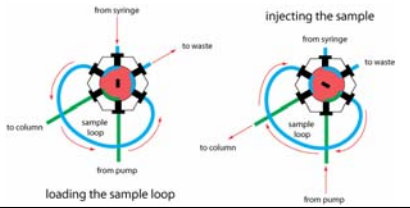
(1) Manual injection

- small volume injection (0.1 to 500 μL) through **sample loop**.
- sampling loop can **control the sample size** of every injection.

(2) Auto sampler

- Many modern HPLC systems are equipped with an **auto-injectors** now.

https://goo.gl/images/JJDwiy



22

9.2 Instrumentation of HPLC

Major components (instrumentation) of a HPLC

★

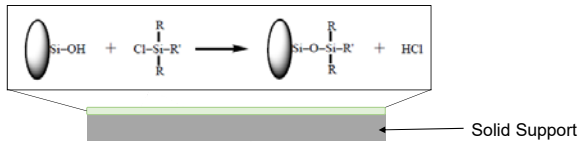
Mobile phase reservoirs → Pumping system → Injector → Column → Detector → Recorder

Sample

23

9.2.4 HPLC columns

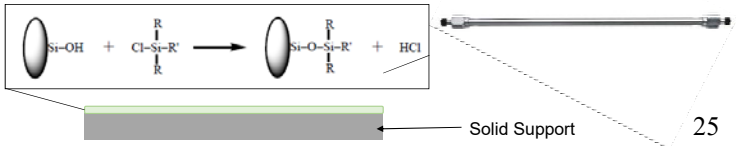
- Different column has different **solid support / stationary phase**
- The stationary phase are prepared by
  - reaction of an organochlorosilane with –OH groups on the surface of silica particles in hot, dilute HCl.



24

9.2.4 HPLC columns

- The R is often ***a straight-chain octyl or octyldecyl group***
  - ***called C8 and C18*** → ***non-polar***
- Other functional groups (R'):
  - aliphatic amines, ethers, nitriles and aromatic hydrocarbons → ***different polarities***
- Sometimes, column is put inside an ***oven*** to control the operation temperature



25

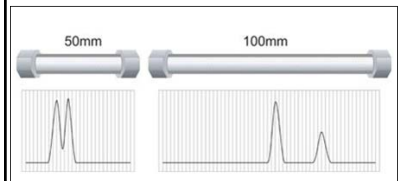
9.2.4 HPLC columns

Improve peak separation / column efficiency

★ **Column length and particle size of stationary phase**

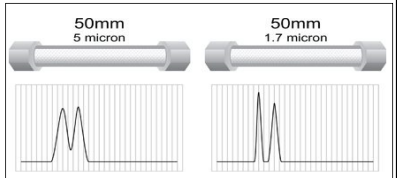
If the column length increase,
- e.g. 50mm→100mm
- **column efficiency will increase**.

If the particle sizes decreases,
- e.g. 5μm → 1.7μm
- **column efficiency will increase**.



Column length and column efficiency
[Same Particle Size]

http://www.waters.com/webassets/cms/category/media/other_images/primer_N_ColumnLength.jpg



Particle size and column efficiency
[Same Column Length]

http://www.waters.com/webassets/cms/category/media/other_images/primer_O_ParticleSize.jpg

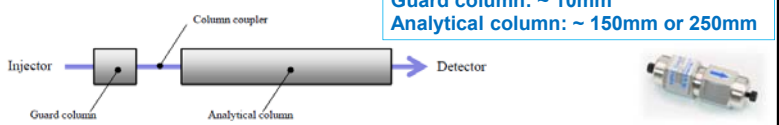
9.2.4 HPLC columns

There's a **guard column** (保護柱子) in front of analytical column:

Property of the guard column:
- **Compositions are same** as the analytical column
  - same stationary phase
  - e.g. analytical column is C18, guard column also is C18

★ **Function of the guard column**:
- **Remove particulate matters / contaminants**
- **Increase shelf-life** of analytical column

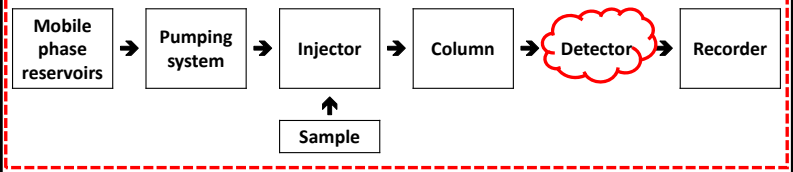
Guard column: ~ 10mm
Analytical column: ~ 150mm or 250mm



9.2 Instrumentation of HPLC

Major components (instrumentation) of a HPLC

★



28

9.2.5 HPLC detectors

Example of HPLC detectors:

Universal detectors	Selective detectors
• Detectors which respond to **all** component	• Detectors which respond to **a related group** of components
For example:	For example:
1. Refractive index (折射率)	1. UV absorbance (紫外線吸收率)
2. Evaporative light scattering detector (ELSD)	2. Fluorescence (螢光)
3. Mass spectrometry (質譜)	

29

9.2.5 HPLC detectors

1. Refractive Index (RI) Detectors 折射率檢測器

- **Comparison** of the refractive index 折射率 of **solvent** (before column) and **eluent** (after column).
- **Difference in refractive index** reflect the **concentration of analyte**.
- ***Characteristics***
  - ➢ **Universal** 通用**detector**
  - ➢ **Not** affected by flow rate
  - ➢ Sensitive to temperature change
  - ➢ **Not** suitable for gradient elution

Diagram of RI detector
https://goo.gl/images/y71ANj

30

9.2.5 HPLC detectors

2. Evaporative Light Scattering Detector (ELSD)

- Column eluent is passed into a **nebulizer** and **converted to fine droplets** by nitrogen flow.
- Fine droplets are then **evaporated** 蒸發 **to remove mobile phase**.
- Measurement is done on **light scattered** 散射
  - (*based on refractive index*) by analyte.

Diagram of ELSD
https://goo.gl/images/bbB8V2

31

9.2.5 HPLC detectors

3. UV Visible Detectors

- Measurement of **UV absorption** based on **Beer's Law**.
- Suitable for samples with **conjugated double bonds** / **aromatic rings**.
- **UV/Vis Detector**: monitors **ONE wavelength** at **one time**.
- **DAD (Diode Array Detector)**: monitors **multiple wavelengths** at **one time**.

Conjugated double bonds

Diagram of DAD
https://goo.gl/images/dcGoW7

Spectra from DAD
https://goo.gl/images/dcGoW7

32

9.2.5 HPLC detectors

4. *Fluorescence Detectors* 熒光

- **Fluorescence emission** 熒光發射 from analyte is detected at **90° to excitation source**.
- High **sensitivity** 靈敏度 and **selectivity** 選擇性 (fewer compounds fluoresce than UV/Vis).
- Needs to **select excitation** and **emission wavelengths**.

Fluorescence emission spectrum

https://goo.gl/images/WM2Pcv

Diagram of fluorescence detector

https://goo.gl/images/t5vHnH

33

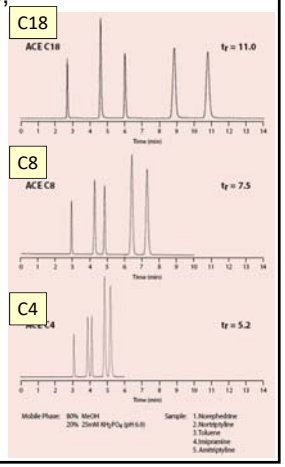
9.3 Columns Selection

Requires a **proper balance** 適當平衡 **of intermolecular forces** among the analyte, mobile and stationary phases.

General steps:

1. Determine the **polarity of analyte functional groups**.
2. To **retain the analyte**, **match the polarity** of stationary phase with that of analyte.
3. A **different polarity mobile phase** is then used for elution.

https://goo.gl/images/AiCn1H

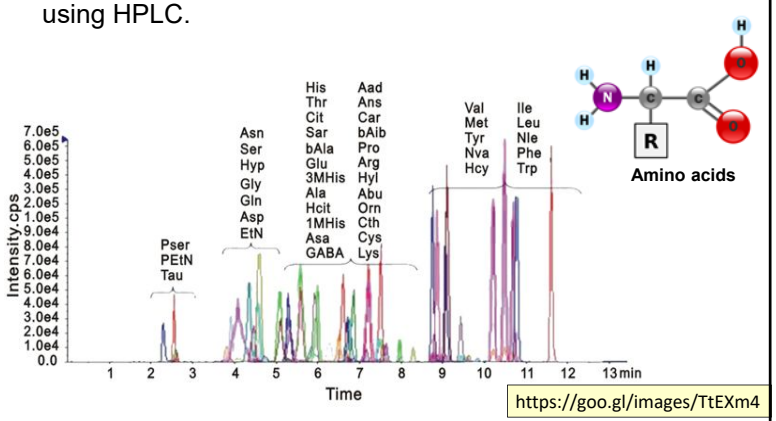


9.4 Applications of HPLC

Reference ONLY!!

Bioscience 生物科學

- Determination of 45 **amino acids** (protein building blocks) using HPLC.



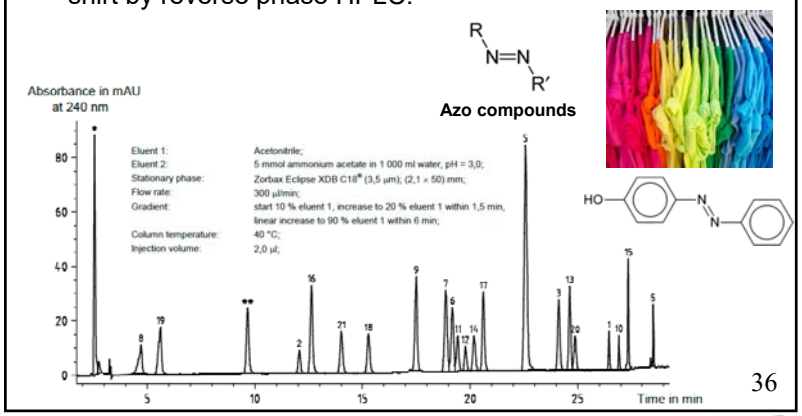
https://goo.gl/images/TtEXm4

9.4 Applications of HPLC

Reference ONLY!!

Consumer products 消費產品

- Determination of carcinogenic 致癌 **azo dyes** 偶氮染料 in T-shirt by reverse phase HPLC.



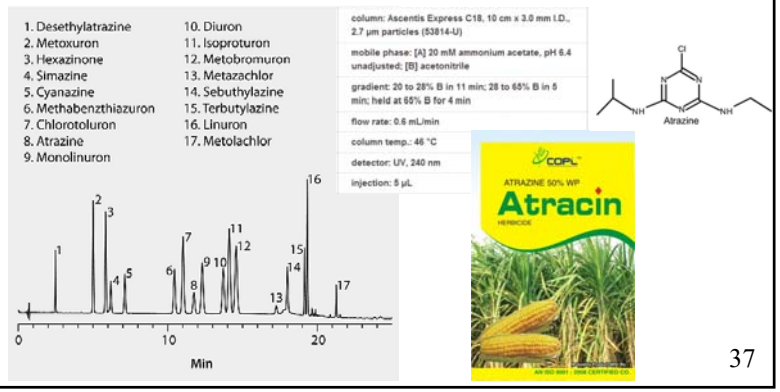
36

9.4 Applications of HPLC

Reference ONLY!!

Environmental

- Determination of **Atrazine Herbicides** (除草劑) in field crops by reverse phase HPLC.



37

Notes

38

Notes

39

Notes

40